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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Characterization of Heterogenous siRNA and Preparation of Superpotent Mixtures

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ENCLOSED APPLICATION PARTS *(check all that apply)*

[X] Specification *Number of Pages* 19
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[ ] Application Data Sheet. See 37 CFR 1.76

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[Page 1 of 1]

Respectfully submitted, SIGNATURE Harriet M. Strimpel

Date 03/09/04

REGISTRATION NO. 37,008 *(If appropriate)*

Docket Number: NEB-240

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Docket No. NEB-240

IN THE UNITED STATES PATENT OFFICE AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTERS PATENT

INVENTOR (S): George Tzertzinis
Celine Petit

TITLE: CHARACTERIZATION OF HETEROGENOUS siRNA AND PREPARATION OF SUPERPOTENT MIXTURES

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Characterization of Heterogenous siRNA and Preparation of Superpotent Mixtures

It is desirable to maximize the efficiency of mixtures of siRNAs in gene silencing as well as to select individual highly effective siRNAs. We obtained highly potent gene silencing mixtures by firstly using an efficient RNaseIII cleavage reaction (US Patent application 20040038278) to convert the long dsRNA into hsiRNA and comparing the silencing activity in an efficient and reproducible biological assay.

We compared the silencing effectiveness of different heterogeneous siRNA (hsiRNA) mixtures targeting the same mRNA sequence, to evaluate the rate of success of obtaining an effective mixture. We varied the targeting region and the size of starting dsRNA, and measured quantitatively the silencing effect at the level of both mRNA and protein knock down.

Firstly the DNA corresponding to the target mRNA of interest was segmented into fragments ranging in size from 60 bp to 600 bp which were converted to hsiRNA using RNaseIII processing as described below. Each hsiRNA mixture was then individually transfected into cells and the silencing effect, at low transfection concentration (1-20 nM), on the target protein and RNA was compared by western blot and quantitative real time PCR.

p53 was used as a model to study RNAi as in previous studies a knock-down with hsiRNA generated from dsRNA 1000 bp-long had been achieved in COS cells (US Pat. App. No 20040038278).

Different constructs were made to span the entire mRNA sequence of p53. Short interfering RNA mixtures were then generated by enzymatic digestion of long dsRNA obtained by in-vitro transcription of each amplified insert. The effectiveness of siRNA mixtures in RNAi of mammalian p53 in cultured cells was measured by western-blotting

with anti-p53 antibody and by real-time PCR after transfection of each mixture. Actin protein and mRNA was used as a non target control.

Transcription template preparation

- Three sets of primers were designed using sequences from the Nucleotide database to cover parts of p53 cDNA sequence not covered by the clones. Each primer contained the T7 promoter sequence and a biotin group was at the 5'end of each forward primer. **Table 1** lists all the primers used for the preparation of p53 constructs.

p53 templates were obtained by using these primers to perform standard PCR on total cDNA from COS-7 cells. The same temperature (68°C) for the three sets of primers was chosen.

After each PCR, a 1% agarose gel was used to check the size of each fragment and the PCR efficiency.

- Templates for *in vitro* transcription from Litmus 28i (**Figure 1**) clones were generated by PCR with biotinylated T7 primer: 5´-Biotin-d(CTCGAGTAATACGACTCACTATAGG) 3´. The PCR was performed with the same conditions as described above with 1 to 10ng of each construct's plasmid DNA.

Purification of transcription templates

Streptavidin-coated magnetic beads were used to purify PCR products in the following steps: adjustment of each PCR reaction at 0.5 NaCl, addition of 50µL of beads in each PCR reaction and binding on the beads for 15min, 3 washes (with TE (10Mm Tris-Hcl pH 8, 1mM EDTA) 0.5M NaCl first and then with TE only) and resuspension in 20µL of TE buffer. A magnetic separation rack was used between each step to separate the beads from the solution.

The PCR fragments immobilized on the beads were used directly for *in vitro* transcription.

In vitro transcription

Transcription reactions were performed in 50µL of transcription mix (Hiscribe kit NEB): 1X transcription buffer (40mM Tris-HCl pH 8.1, 19mM $MgCl_2$, 5mM DTT, 1mM spermidine, 4mM each NTPs), 1X High Molecular Weight Mix (0.67mM Tris-HCl pH 8.1, 0.05 mg/mL BSA, 0.2 U inorganic pyrophosphate from yeast, 24 U pancreatic ribonuclease inhibitor, 2% glycerol), 300 U T7 RNA polymerase and the DNA template coated on 20µL resuspended beads. After 2h incubation at 42°C, an annealing protocol was performed for 3 min at 72°C, followed by 3 min at 68°C, 5 min at 65°C, 10min at 62°C and a final step at 42°C for 15 min. The magnetic beads were removed by magnetic separation and the mixtures were diluted two fold with water, then adjusted to 0.3 M sodium acetate pH 5.2, and precipitated with 2 volumes of cold 95% ethanol overnight at -20°C. After 15min of centifugation at 14000 rpm, the pellet was washed once with 70 % ethanol, allowed 15min to air dry and resuspended in 50µL of water. The RNA was dissolved by 5 min incubation at 70°C and 10 min at room temperature.

RNase III digestion

Before the digestion, dsRNA concentration was determined on 1% agarose gel (Fig.1) by comparison to DNA standards by using a suitable software package (Quantity One from Biorad). To prepare heterogeneous mixtures of siRNA, 10µg of dsRNA were digested in a 100µL reaction buffer: 1X Short Cut reaction buffer (0.05 M Tris-HCl pH 7.5, 1mM DTT), 20mM $MnCl_2$, 13 U RNaseIII for 20min at 37°C. Reactions were terminated by adding EDTA to 25 mM in each tube. 10µL of each digestion were loaded on a TBE 20% gel (non denaturing

gel) to quantify the digestion products by comparison to a 21bp si RNA marker. After ethanol precipitation in the presence of 20 µg of glycogen, the samples were resuspended in 30µL of water.

For hsiRNA mixtures, the average molecular weight is : 2 x 22 x 361 = 15884 g/mol (361 is the average molecular weight of the 4 RNA bases). For example, to obtain a molarity of 10nM in a transfection performed in 0.3mL (24 wells plate) the calculation was:

ng x (1nmol / 15884 ng) x 1 / $0.3x10^{-3}$ L = 10 nM , which gives 47,65 ng per transfection well.

Cell culture and hsiRNA transfections

COS-7 african green monkey kidney cells were cultured at 37°C in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% heat-inactivated FBS, 100U/mL penicillin and 100µg/mL streptomycin (Life Technologies).

Cells were passaged every three days by trypsinizing them and diluted them in fresh complete growth medium. Twenty-four hours before transfection the cells were splitted.

To silence p53 expression, mammalian cells were transfected with different amounts of siRNA by using Trans-ITKO transfection reagent (Mirus) according to the manufacturer's instructions.

Cells were transfected at 50-70% confluence in 12 or 24-well plates containing respectively 1mL or 0.5 mL of medium per weel. Transfection reagent (3µL for a 24-weels plate) was mixed with serum-free media (50µL) and incubated for 10min. The complexation was performed for 10min after addition of the appropriate amount of siRNA to the previous mixture. Complete fresh growth medium (250µL) was then added to each tube and 300µL were aliquoted in

each well after aspirating the old medium from each weel. Cells were grown at 37°C for 48h and then washed two times with cold BPS.
Transfection efficiency was determined by fluorescence microscopy of cells transfected with a fluorescein-labeled synthetic short RNA (NEB).The cells were visualised using a motorized Carl Zeiss inverted microscope Axiovert 200M for transmitted light and epifluorescence with 20X or 40X objective lens.

Preparation of cell extracts and Western Blotting

Cells were lysed on ice with a 1% Triton-X, 50 mM Tris-HCl, 150 mM NaCl Buffer, and after centrifugation of the cell lysates at 14000 rpm, supernatants were stored at -20°C.
Equal amounts of protein (10µg) from each transfected well (determined by BCA protein assay (Pierce)) was resolved by 10-20% polyacrylamide Tris-glycine gel (Novex) in SDS buffer (125 mM Tris, 1M glycine, 20 mM SDS) at 30 mA constant for 1h. A prestained protein marker was loaded to monitor protein migration in the gel and blotting, and a biotinylated one to visualise the size of proteins after development. Then, transfer to a PVDF membrane (Millipore Immobilon-P transfer membrane 0.45µm) was performed by electroblotting for 2h at 30V constant in transfer buffer (25 mM Tris base, 0.2 M glycine, 10% MeOH). Staining of the membrane in Poinceau red (0.1%) was used to evaluate the transfer. The membrane was blocked in Blocking buffer (1X TBS: 20 mM Tris base, 136 mM NaCl; 0.15% Tween; 5% w/v non-fat dry milk). After blocking, two different antibodies were used: a mouse monoclonal anti-p53 (CST) diluted 1/1000 and a rabbit polyclonal anti-actin (Sigma) dilution 1/3000 as a control. Immune complexes were visualised with the Phototope-HRP (horseradish peroxidase) Western

Blot Detection System containing the anti-rabbit HRP-conjugated secondary antibodies, a biotinylated marker and the detection reagent LumiGlo (CST). Three Washes in TBS 1X, 0.15% Tween were made between steps. When necessary stripping of the membrane (Restore Western Blot Stripping Buffer (PIERCE) was performed after the detection of p53. The membrane was then reprobed with anti-actin or other antibodies.

Preparation of total RNA

The kit RNA aqueous (Ambion) was used to prepare total RNA from crude cell extracts.

The samples were lysed in 350µL per well (for 12-well plates) of Lysis and the RNA was eluted in 50µL of the low ionic strength elution solution.

Before cDNA synthesis, the quality and amount of RNA was determined by spectrophotometry at 260nm and evaluated in 1% agarose gel, in formamide loading buffer.

cDNA synthesis

The synthesis of cDNA was performed in 20µL reactions according to the instructions of the Protoscript first strand cDNA synthesis kit (NEB) using approximately 0.5 to 1µg of the extracted total RNA and the reaction was diluted to 50µL in water.

After each synthesis, a standard PCR with 0.2µM GAPDH primers was performed to verify the efficiency of synthesis and visualise any differences.

Real-time PCR assays

Different sets of specific primers were designed and optimized: one for p53, one for 28S and one for beta-actin. The annealing temperature was 60°C. The sequences of these primers and their position on each cDNA sequence are indicated on **Table 2** and in **Figure 2** respectively (for the p53).

The reactions were performed in 50µL total volume containing 1X final SYBR green supermix (BioRad) : 50mM KCl, 20mM Tris HCl pH 8.4, 0.2 mM each dNTP, 3 mM $MgCl_2$, 10 nM fluorescein, 1.25 U iTaq DNA polymerase, SYBR Green I; 0.2 µM of primers and 2µL (1/25 of total cDNA volume) of cDNA.

A master mix was prepared for each set of primers and aliquoted in each well, before the DNA samples. The plate was pulse-centrifuged at 1000rpm.The following PCR program was performed on the i-Cycler machine (BioRad) : 95°C for 5min, 30s 95°c, 30s 60°C for 40 to 50 cycles, 95°C for 1min. The data for the melting curves were acquired every 0.5°C from 40°C to 95°C (110 steps).

The results were expressed using the threshold cycle (Ct) which was set at the point in which the signal generated from the sample is significantly greater than background fluorescence.The knock-down of p53 was evaluated relative to beta-actin as the reference gene.

The formula used for each sample was: $2^{(-(Ct\ p53-Ct\ actin))}$ where Ct p53 is the threshold cycle for amplification of p53 and Ct actin is the cycle threshold for the actin (Heil et al., 2003).

The knock-down was calculated as a fraction of the relative expression of RNAi samples to relative expression of the control (cells transfected with a siRNA mixture targeting another mRNA) which was set to 100%.

Name and position on human p53 cDNA sequence	Designed Primers
P 2 128-712	**Forward primer** 5'- BCTCGAGTAATACGACTCACTATAGGCCGTCCAGGGAGCAGGTAGCT-3' **Reverse primer** 5'- CTCGAGTAATACGACTCACTATAGGCCGGGCGGGGGTGTGGAATC-3'
P 3 1428-2021	**Forward primer** 5'- BCTCGAGTAATACGACTCACTATAGGGACTGACATTCTCCACTTCTT-3' **Reverse primer** 5'- CTCGAGTAATACGACTCACTATAGGCTCTACCTAACCAGCTGCCCAACT-3'
P 4 1968-2567	**Forward primer** 5'- BCTCGAGTAATACGACTCACTATAGGCCTGTTGGCTGGTGGGTTGGTAGT-3' **Reverse primer** 5'- CTCGAGTAATACGACTCACTATAGGAGGAGGGGAAGGGTGGGGTGAAAA-3'

Table 1 : PCR primers designed from human p53 cDNA sequence The coordinates for the position of each amplified fragment are shown on the human p53 cDNA sequence (gi 8400737) in nucleotide number (B = biotin)

	Primers	Position on cDNA sequence and size of the amplicon
Beta-actin real-time PCR primers	**Forward primer** TGCGTGACATTAAGGAGAAG **Reverse primer** GCTCGTAGCTCTTCTCCA	**Size:** 98 bp **Position:** Human sequence (gi 5016088):699-797 African Green Monkey sequence (gi 2116654): 636-734
p 53 real-time PCR primers	**Forward primer** CCCCCTCCTGGCCCCTGTCATCTT **Reverse primer** CGGGCGGGGGTGTGGAATCAAC	**Size:** 197 bp **Position:** Human sequence (gi 8400737):514-711 African Green monkey sequence (gi 22795): 365-562

Table 2: Primers used for real-time PCR assay and their position on each corresponding Human and Green African Monkey cDNA sequence.

Different AciI restriction fragments of p53 human cDNA were cloned in Litmus 28i Vector, at the BstBI site. After sequencing each plasmid DNA subclone, the position of each on the p53 human cDNA sequence was determined by BLAST 2 sequences alignment. Alignment of human and monkey p53 cDNA sequence showed over 95% of homology so the use COS-cells instead of human cells for the experiments did not pose a problem. The constructs were obtained from the human sequence.

- After determining the position of each construct, three sets of PCR primers were designed for the remaining parts by using p53 human cDNA sequence from Nucleotide database in order to cover the entire sequence of p53. Since the entire p53 cDNA sequence from Green African Monkey (COS-cells) was not in the database, the (5' and 3' segments were missing) we used the human sequence to obtain remaining parts by PCR using the primers shown in Table 1. These same primers were used to obtain the missing regions by PCR amplification from COS cells cDNA. After purification, the PCR products obtained with each primer set were sequenced and aligned with human sequence to determine differences between human and monkey sequence. The percentage of homology was between 96 and 98% for the three new parts amplified in these conditions.

Thus the complete sequence of p53 mRNA (2.6 kb) was covered by cloned and amplified fragments exept the first 128 nucleotides and the 60 last ones (**Figure 2**). The size of each cloned or amplified fragment varied from a minimum of 66 to approximately 600 bp. Some of the clones (C6, C9, C14, C20) contained non contiguous segments of the p53 sequence.

Comparison of siRNA mixtures effectiveness by Western-Blotting

In order to qualitatively compare the effectiveness of the different siRNA mixtures in RNAi, the transfected cell lysates were studied by Western-Blotting after 36 to 48h of transfection. The knock-down of p53 was evaluated by comparing the expression level of p53 in cells transfected with control siRNA mixtures targeting another mRNA and cells transfected with siRNAs mixtures targeting p53. The different mixtures were used between 10nM and 25 nM to allow comparisons because of previous results that show a knock-down at these concentrations or above.

On all the western-blots (**figures 3 to 5**) a band at 53kDa correspondsing to p53 protein is detected. The control lanes have a strong band for p53 expression that is higher than in the other lanes. However, since this difference of expression can be due to differences of protein amount loaded in each lane, another probing of the membrane was performed with a control antibody to obtain a ratio between p53 and control protein levels to be able to compare semiquantitatively the results between clones.

In most cases the expression level of control protein was almost the same in all the different lanes whereas differences in p53 expression were observed.

All mixtures used at 10nM lead to a significant decrease of p53 (**Figures 3 and 4**) since the level of p53 in the samples with siRNA mixtures targeting p53 is lower than in the control and because the level of actin is equal between the samples and the control.

A ratio of p53:actin was sometimes calculated after scanning of the film to obtain an approximation of the p53 knock-down and the results are shown in **Table 3**. This calculation shows the decrease of p53 expression level after transfection of the cells by heterogeneous siRNA mixtures. The western-blot on **figure 4** shows that the P3 and P4 gave a significant knock-down whether they are used at 10nM or 25nM.

In order to evaluate the differences between the transfected concentration of siRNA mixtures in a broaders range of concentration, a titration from 1.5nM to 150nM was performed with the siRNA mixture prepared from construct C2. Significant knock-down is already visible at 10nM. Above a certain threshold, between 15 and 30nM, the knock-down obtained with the hsiRNA mixture is higher. However, this increase is not linearly proportional to the concentration. The first three concentrations used (from 1.5 nM to 15 nM) seem to cause a similar level of knock-down. Above 15 nM a more significant decrease in p53 level is detectable and no significant difference can be seen between the results obtained with the different concentrations above 15 nM. Scanning and quantitation of the Western-blot shows a decrease from 50 to 60% for the three lower concentrations of siRNA and sthe knock-down obtained with concentration above reached 80 to 85% (**Table 3**). This level of p53 could easily reflect background from untransfected cells since the transfection efficiency of fluorescent siRNA was estimated to be 85-95%.

Another experiment was performed to visualise the duration of the protein silencing effect. The western-blot (**Figure 5**) shows a silencing effect after 48h of transfection which lasts until more than 4 days after transfection.

Table 3:

Samples	Relative expression (%)
Control (creb)	100
C2 1.5 nM	50.1
C2 7.5 nM	38
C2 15 nM	38.8
C2 30 nM	17
C2 75 nM	17
C2 150 nM	21

Table listing the expression of p53 calculated by the ratio between p53 and actin, and relatively to the control treatment (hsiRNA against CREB) which was set to 100%.

The results obtained for the study of the effectiveness of different mixtures by real time PCR are represented in **figure 6** ordered per position on the p53 cDNA sequence. The histogram shows the relative expression of p53 (in percent) calculated by using the threshold cycle values obtained for p53 and actin and by comparing the experimental samples to a control (non specific hsiRNA). The differents samples were studied several times with an average standard deviation of 3.6%.

All the constructs used at 10 nM lead to a knock-down of target mRNA between 64 to 92% which indicates that all the siRNA mixtures used are efficient in RNAi of p53 (**Figure 6**).

Comparing the results obtained with all themixtures, shows that there is no obvious correlation between the effectiveness and the position of the constructs, for example C2 and P3 have almost the same effectiveness although C2 is in the coding region and P3 is in the 3′ untranslated region. Additionally there is no obvious correlation with

the size of the constructs used for the mixtures, for example P2 and P4 which are the longest fragments (approximately 600 bp) lead to similar knock-down levels of 20% as do C1 (66 bp) and C10 (133 bp) which are the shortest fragments.

According to all these results, it seemed that there is no real region-dependence when using such mixtures.
Importantly an exceptional knock-down, of over 90%, is obtained when using the mixtures prepared from the templates with the non contiguous pieces C6, C14, C20. It is difficult to evaluate differences on the effectiveness between these particular mixtures, since 10 nM already causes the maximmum possible knock down, it may be possible that a much lower transfected concentration could be suficient for effective knock downs .
The titration with C2 siRNA mixture from 1.5nM to 150nM (**Figure 7**) shows a significant knock-down even when the mixture is used at the lowest concentration of 1.5 nM.
Although synthetic siRNAs and also plasmid generating siRNAs that can effectively suppress the expression of endogeneous gene have been reported ((Elbashir et al., 2001a)), the efficiency of such siRNAs is highly dependent on target position ((Holen et al., 2002)). Therefore, the limiting factor was the design and cost of efficient. Therefore, the limiting factor was the design of efficient siRNAs as it costs a lot of time and money.

Our data obtained with Western-blot analysis or real-time PCR assays were comparable and lead to the conclusion that unlike single sequence siRNAs, there are multiple possible mixtures against a target

mRNA that have a good RNAi efficiency. This could be explained by the fact that siRNA mixtures target multiple sites within the mRNA.
The size and the position can vary as well without affecting the RNAi efficiency, for example C1 (66 bp) is as effective as P3 (600 bp) when they are used at 10nM.
The titration performed with one of the mixtures allow to conclude that there is a concentration dependence, a threshold (between 15 and 30nM) above which the silencing effect is not increase. In previous studies the standard siRNA concentration was above 20nM ((Kawasaki et al., 2003); (Elbashir et al., 2001a)) but if the concentration is too high it can provoke non-specific response (Semizarov et al. 2003).
Another interesting result is that the effectiveness of siRNA mixtures from non-contiguous pieces, except for the clone 9, lead to a more significant knock-down which is above 90%. The efficiency of these super-potent mixtures in RNAi is detectable either qualitatively or quantitatively and could be the result of a sampling of different potent regions.

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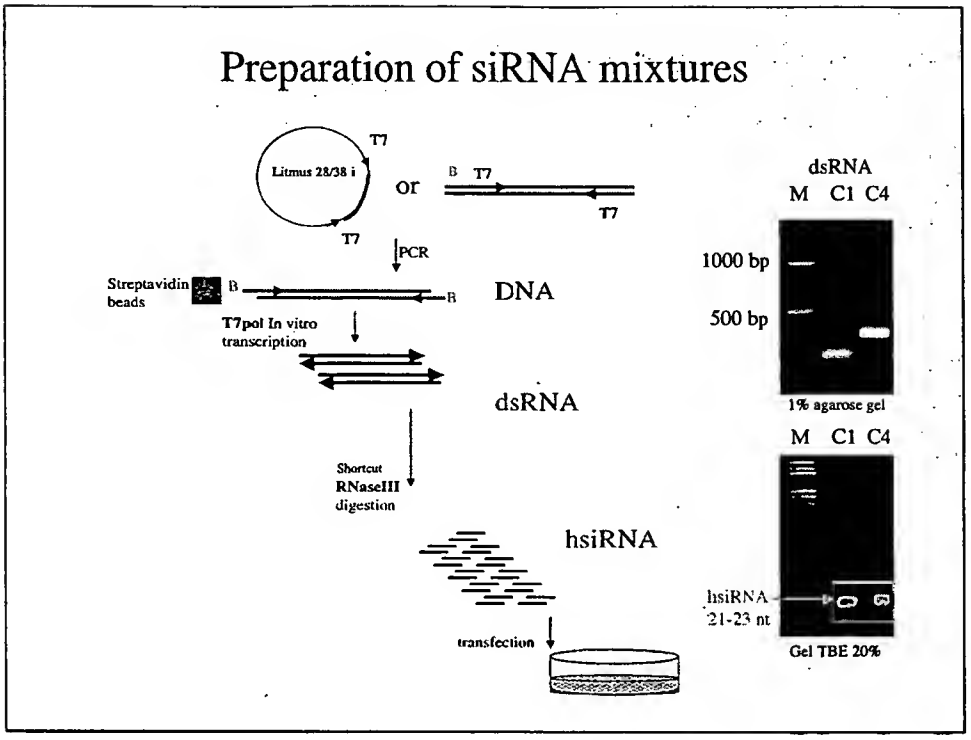

Fig. 1

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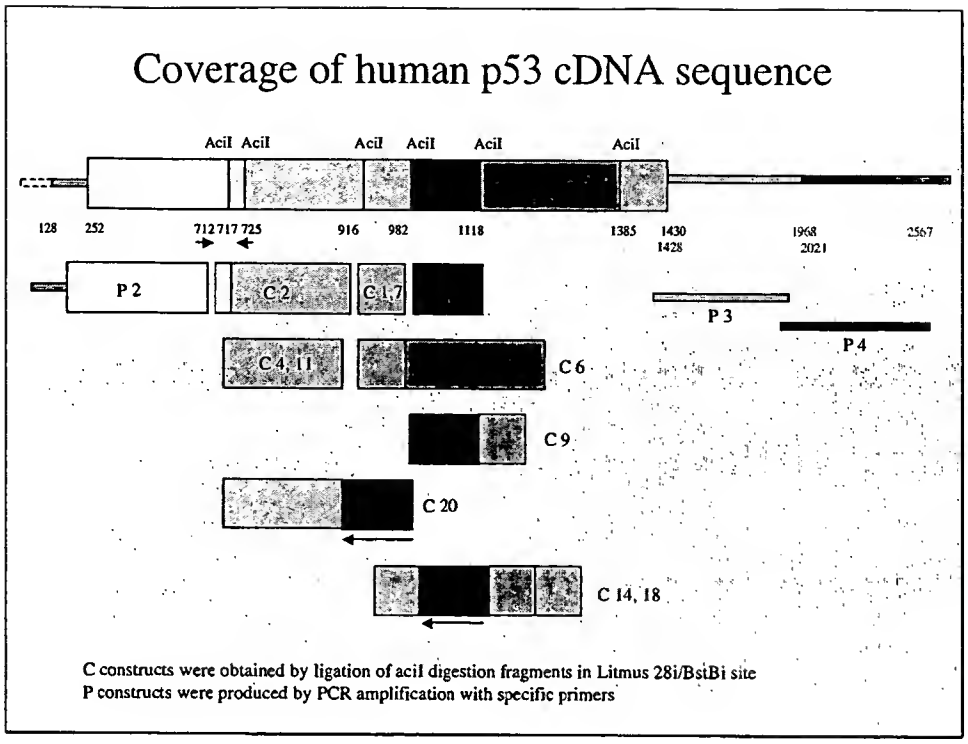

FIG. 2

C constructs were obtained by ligation of aciI digestion fragments in Litmus 28i/BstBi site

P constructs were produced by PCR amplification with specific primers

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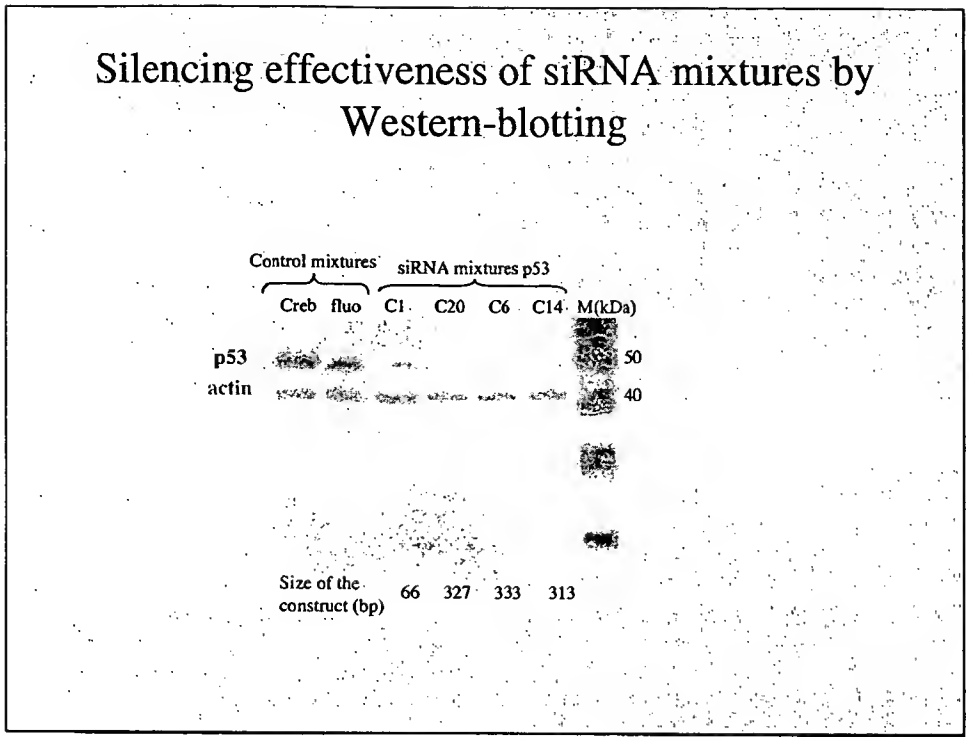

Figure 3

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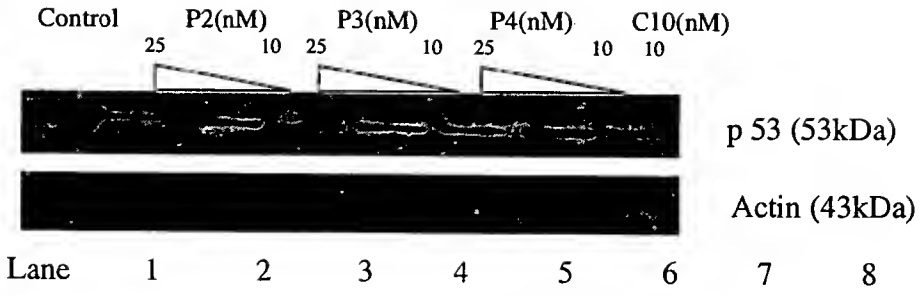

Figure 4 : Western-Blot analysis of the expression of p53 and beta-actin
Lane 1: 10µg of protein from cells transfected with a mixture targeting another mRNA
Lanes 2 to 8: 10µg of protein from cells transfected with different siRNA mixtures targeting p53 at different concentration

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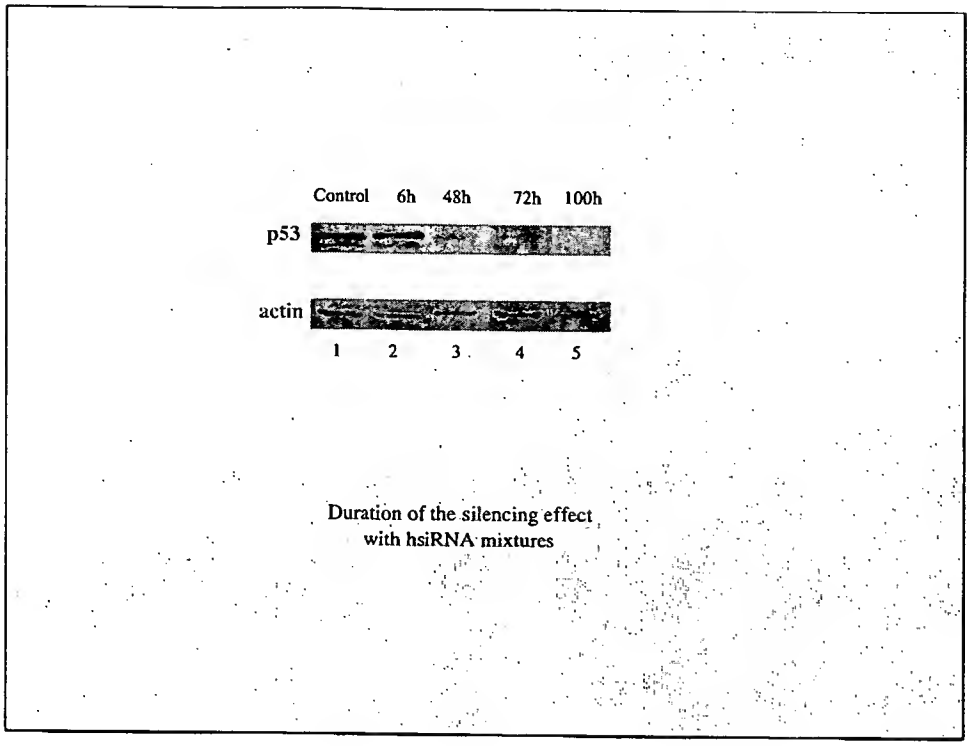

Figure 5

Figure 5: Duration of siRNA-mediated silencing effect

Lane 1: control with a siRNA mixture targeting another mRNA, (control samples for each time-course point were all equal in intensity)

Lane 2 to 5: study of p53 and beta-actin expression between 6h and 100h. 8μg of total protein from cells transfected with siRNA mixture C2 at 25 nM

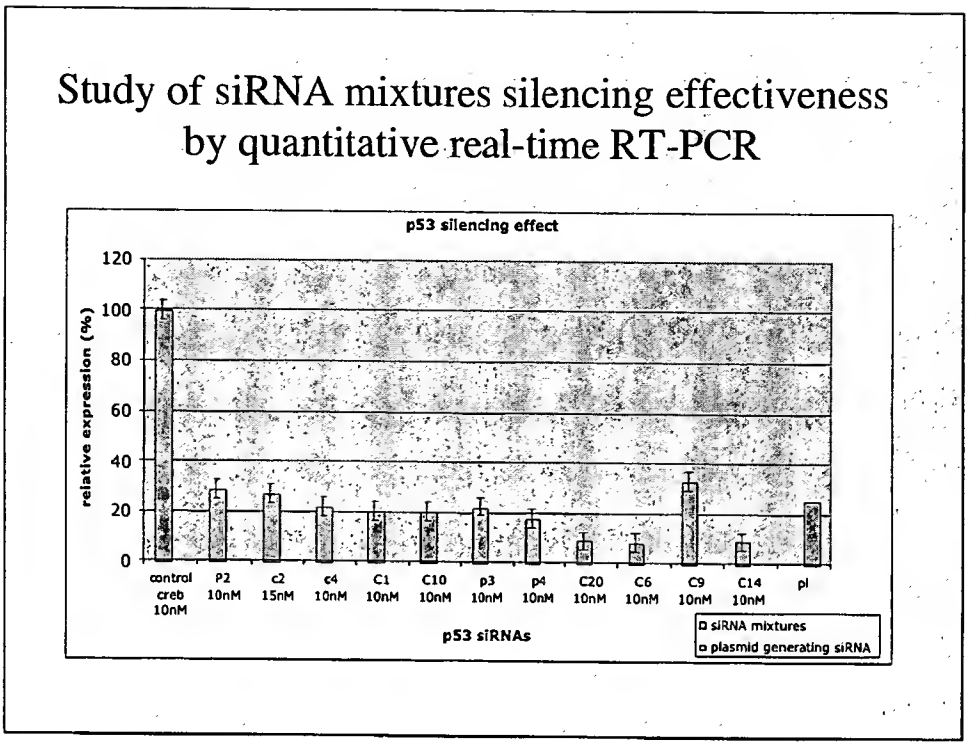

Figure 6

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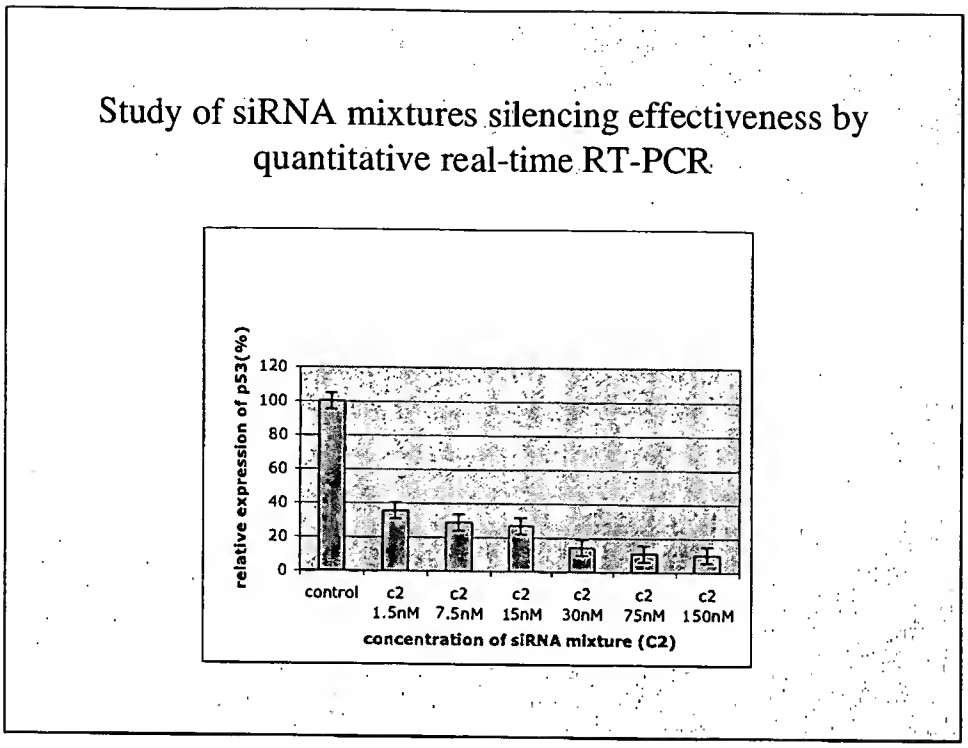

Figure 7

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